

Research Paper

Lipase/acyltransferase-catalysed interesterification of fat blends containing *n*-3 polyunsaturated fatty acids

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The lipase/acyltransferase from *Candida parapsilosis* is an original biocatalyst that preferentially catalyses alcoholysis over hydrolysis in biphasic aqueous/organic media. In this study, the performance of the immobilised biocatalyst in the interesterification in solvent-free media of fat blends rich in *n*-3 polyunsaturated fatty acids (*n*-3 PUFA) was investigated. The interesterification activity of this biocatalyst at a water activity (a_w) of 0.97 was similar to that of commercial immobilised lipases at a_w values lower than 0.5. Thus, the biocatalyst was further used at an a_w of 0.97. Response surface modelling of interesterification was carried out as a function of medium formulation, reaction temperature (55–75 °C) and time (30–120 min). Reaction media were blends of palm stearin (PS), palm kernel oil and triacylglycerols (TAG) rich in *n*-3 PUFA (“EPAX 4510TG”; EPAX AS, Norway). The best results in terms of decrease in solid fat content were observed for longer reaction time (>80 min), lower temperature (55–65 °C), higher “EPAX 4510TG” content and lower PS concentration. Reactions at higher temperature led to final interesterified fat blends with lower free fatty acid contents. TAG with high equivalent carbon number (ECN) were consumed while acylglycerols of lower ECN were produced.

Keywords: *Candida parapsilosis* lipase/acyltransferase / Interesterification / Modelling / *n*-3 Polyunsaturated fatty acids / Response surface methodology

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1 Introduction

In the last decade, the production of new lipids with functional/nutraceutical properties, *i.e.* “structured lipids” (SL) or “tailor-made fats”, has greatly increased. SL consist of triacylglycerols (TAG) that have been either (i) modified by the incorporation of new fatty acids, (ii) restructured to change the region-distribution of fatty acids or (iii) synthesised to yield novel TAG [1]. These modified fats with novel properties present important medical, nutraceutical and food applications [1–4].

SL enriched in *n*-3 polyunsaturated fatty acids (*n*-3 PUFA), especially in eicosapentaenoic acid [EPA, 20:5 (*n*-3)] and docosahexaenoic acid [DHA, 22:6 (*n*-3)] have a great potential due to their benefits in human health, namely the prevention and treatment of heart diseases, by increasing high-density lipoprotein cholesterol levels in serum, and the reduction of inflammatory conditions [1]. The incorporation of *n*-3 PUFA in food products more readily available for consumption than marine fat fish, such as vegetable oils, dressings and margarines, may be an interesting option to the human diet.

Prior to their incorporation in margarines, shortenings or in other fat products, the improvement of certain physical properties of fat blends [*e.g.* melting point, solid fat content (SFC) and crystallisation pattern] is frequently achieved by interesterification reaction (ester interchange), where the modification of their acylglycerol pattern occurs. Thus, in

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these SL, the original fatty acid composition is maintained and no *trans* fatty acids are produced, in contrast to what happens when the physical properties of fat blends are modified by hydrogenation [5]. The production of *trans* acid-free alternatives for margarines and other food products, together with the incorporation of *n*-3 PUFA in these products, has been an increasing market trend.

In the food industry, chemical interesterification of fat blends is currently carried out at temperatures ranging from 50 to 120 °C, for less than 2 h, using metal alkylates or alkali metals (e.g. sodium methoxide and sodium) as catalysts. The interchange of acyl groups proceeds at random. In addition, the final products may remain contaminated by residual catalyst, and the formation of considerable amounts of side products, with a subsequent decrease in yield, may occur [5].

In the context of the growing consumer demand for natural and healthy foods, the replacement of chemical catalysts by biocatalysts, recognised as natural and therefore leading to the synthesis of natural and healthy products, has become a challenge for the food industry. With respect to the technology of oils and fats, the replacement of inorganic catalysts by enzymes (e.g. lipases) is highly desirable, due to the benefits of the enzymatic route relative to chemical processes.

Lipases (acylglycerol acylhydrolases, EC 3.1.1.3.) are considered as hydrolases that catalyse the hydrolysis of water-insoluble esters at water/oil interfaces. Most of them exhibit an interfacial catalytic kinetics [6]. In addition, lipases are also able to catalyse different reactions, namely esterification of free fatty acids (FFA), alcoholysis of a donor ester (transesterification), and combined reactions resulting in the exchange of acyl groups between two esters (interesterification) or between an ester and an FFA (acidolysis). In general, these reactions are performed in non-aqueous media under low-water activity (a_w) conditions in order to limit the hydrolysis side reaction [7].

SL can be obtained by acidolysis, interesterification or esterification, catalysed either chemically or enzymatically. The synthesis of SL containing *n*-3 PUFA, catalysed by lipases, has been reported, namely by acidolysis with free *n*-3 PUFA or interesterification with methyl ester forms of *n*-3 PUFA or with concentrates of TAG rich in *n*-3 PUFA, either in organic media or in solvent-free media [8–10].

The mechanism of lipase-catalysed interesterification involves the hydrolysis of ester bonds in TAG followed by re-esterification [11–13]. Thus, the optimisation of interesterification reactions results from a balance between the rates of hydrolytic and esterification reactions. However, this is not always easy to achieve because, when water is in excess, a displacement towards hydrolysis is observed. Conversely, the global reaction is shifted towards esterification when low water amounts are present. The first interesterification stage should be conducted at a water activity close to 1, while the second stage should be carried out at lower values [14].

In the esterification and interesterification of lipids in non-aqueous systems, the highest activity of the majority of lipases

is usually observed at a_w values between 0.20 and 0.50, or even under extremely dry conditions (a_w from 0.064 to 0.11), depending both on the biocatalyst and on the reaction itself [11, 15–21].

However, some lipases present their maximum of esterification and interesterification activities at a_w values higher than 0.5 and in some cases close to 1, under conditions where the hydrolysis reaction is supposed to occur [20, 22, 23].

In addition, lipase-catalysed synthesis of esters by esterification or alcoholysis is also reported to occur in aqueous media. That is the case of the lipase/acyltransferase from *Candida parapsilosis* that, when in aqueous or in biphasic aqueous/organic media, preferentially catalyses alcoholysis over hydrolysis, whereas other lipases such as that from *C. deformans*, *Rhizomucor miehei* or *Rhizopus delemar* are able to catalyse ester production by direct esterification in aqueous media [24–29].

This study aims at the search for biocatalysts with eventual novel properties, as an alternative to the commercial immobilised lipases used in the majority of interesterification studies. The performance of the immobilised *C. parapsilosis* lipase/acyltransferase as catalyst for the interesterification of fat blends rich in *n*-3 PUFA, in solvent-free media, was investigated. The present work deals with the production of SL prepared from blends of palm stearin (PS), palm kernel oil (PK) and concentrates of TAG rich in *n*-3 PUFA, for use in nutraceutical applications, particularly as margarine fat bases. From the industrial point of view, by interesterification of fat blends, it is expected to reduce the original amount of crystallised fat (SFC) at storage, processing and consumption temperatures.

The effect of the initial a_w of the biocatalyst on the interesterification kinetics was evaluated. Also, modelling the interesterification and optimisation of reaction conditions (medium formulation, reaction time and temperature) by response surface methodology was attempted. The modification of the acylglycerol profile and the accumulation of oxidation products and FFA throughout the enzymatic interesterification were also assessed.

2 Materials and methods

2.1 Materials

Refined, bleached and deodorised PS and PK were supplied by FIMA/VG, Produtos Alimentares, Portugal. The commercial concentrate of TAG rich in *n*-3 PUFA, “EPAX 4510TG” (45% EPA and 10% DHA), was a gift from EPAX AS, Lysaker, Norway.

The lipase/acyltransferase from *C. parapsilosis* was produced by overexpression of the corresponding gene in *Pichia pastoris* as described by Brunel *et al.* [30]. Acetone HPLC grade, from Fisher Scientific, UK, and acetonitrile for HPLC, gradient grade, from Sigma-Aldrich, Germany, were used.

The other reagents used were of *p.a.* grade and obtained from various sources.

2.2 Methods

2.2.1 Enzyme immobilisation

C. parapsilosis lipase/acyltransferase was immobilised on Accurel MP 1000 (Membrana GmbH, Obernburg, Germany) as follows. A 1-g sample of dry immobilisation support was wetted in 7 mL ethanol for 15 min under gentle magnetic stirring at about 20 °C. After ethanol removal by suction, 20 mL enzyme solution (100 mg enzyme) was added to the wet support and the suspension was gently stirred for 24 h at room temperature. The biocatalyst was then stabilised by covalent reticulation. For this, 25 mL of a 2.5% (vol/vol) glutaraldehyde solution was added to the immobilisation medium and left for two more hours under stirring at about 20 °C. The supernatant was then removed by suction over a glass filter and the immobilised biocatalyst was washed six times with 10 mL 20 mM sodium phosphate buffer, pH 6.5. The liquid phase was finally removed; then the immobilised enzyme was kept at 4 °C before use.

2.2.2 Interesterification using the biocatalyst at different initial water activities

The immobilised biocatalyst was pre-equilibrated, for 4–5 days at 30 °C, with the vapour phase of the following saturated salt solutions of known a_w : K_2CO_3 ($a_w = 0.4317$); KI ($a_w = 0.68$); $Mg(NO_3)_2$ ($a_w = 0.514$) and $(NH_4)_2SO_4$ ($a_w = 0.8$) [31]. The final a_w was measured in a Rotronic Hygroskop DT humidity sensor (DMS-100H), at 30 °C. The immobilised lipase at each pre-established a_w value was used in batch interesterification reactions.

Interesterification reactions were performed in thermostatted cylindrical batch reactors, at 60 °C, under magnetic stirring, for 24 h. The reaction medium consisted of a ternary blend of 65% PS, 20% PK and 15% “EPAX 4510TG” (wt/wt). A load of 5 wt-% of the immobilised lipase/acyltransferase was added to the reaction medium, after complete melting. Prior to and during each experiment, 5-mL samples were taken and the biocatalyst was removed by paper filtration at approximately 70 °C. All samples were stored at –18 °C for subsequent analysis.

2.2.3 Modelling enzymatic interesterification experiments

Interesterification reactions were performed as described above (Section 2.2.2).

The best reaction conditions were established *via* response surface methodology [32, 33]. The 27 interesterification experiments were carried out following a central composite rotatable design (CCRD), as a function of four factors: reac-

tion medium formulation (PS and “EPAX 4510TG” concentrations), temperature and reaction time. The proportions of the three fats in the reaction medium (PS, PK and “EPAX 4510TG”) varied as dictated by the experimental design. Thus, PS varied from 45 to 85%, “EPAX 4510TG” from 5 to 25 wt-%, and PK from 0 to 40%. Reaction temperature varied from 55 to 75 °C, and reaction time from 30 to 120 min.

A load of 5 wt-% of the immobilised lipase/acyltransferase, at its original water activity (0.97), was used. At the end of each experiment, 10-mL samples were taken and the enzyme was separated from the medium by paper filtration in an oven at approximately 70 °C. All samples were stored at –18 °C for subsequent analysis.

2.2.4 Analytical methods

2.2.4.1 Solid fat content assay

As in the food industry, the time course of the interesterification of fat blends was indirectly followed by the assay for the amount of the solid fraction at different temperatures, known as SFC, by nuclear magnetic resonance (NMR). The SFC values at 10, 20 and 30 °C ($SFC_{10^\circ C}$, $SFC_{20^\circ C}$, $SFC_{30^\circ C}$) are related to the rheological behaviour of fats during storage, packaging and utilisation of bakery margarines, respectively. The SFC at 35 °C ($SFC_{35^\circ C}$) is particularly important for table margarines since it is related to the extent of melting in the mouth. $SFC_{35^\circ C}$ values of the interesterified fats must be smaller than their original counterparts, and as low as possible, to prevent a sandy and coarse texture of the margarine.

The SFC at 10, 20, 30 and 35 °C ($SFC_{10^\circ C}$, $SFC_{20^\circ C}$, $SFC_{30^\circ C}$ and $SFC_{35^\circ C}$) of the blends were assayed in a pulsed NMR spectrometer (Minispec P-20i, IBM). For NMR analysis, samples were melted at 60 °C, maintained at this temperature for about 10 min, then kept at 0 °C for 60 min and finally maintained for 30 min at the test temperature prior to the SFC measurement [34].

2.2.4.2 Free fatty acid assay

FFA content was assayed by titration with a 0.1 N sodium hydroxide aqueous solution. FFA percentage (wt/wt) was calculated on the basis of the molecular weight of oleic acid.

2.2.4.3 Oxidation product assay

Thermal oxidation of the fat was indirectly evaluated by UV absorbance at 232 nm, Abs_{232nm} (related to the presence of initial products of oxidation, *i.e.* conjugated hydroperoxides), and at 270 nm, Abs_{270nm} (final oxidation products, *i.e.* FFA, aldehydes and ketones), of 1% (wt/vol) fat blend in *iso*-octane.

2.2.4.4 Assay for acylglycerol profile

Changes in acylglycerol profile, occurring by the inter-esterification reaction, were evaluated by non-aqueous reverse-phase high-performance liquid chromatography (HPLC) using a Merck Hitachi (Germany) chromatograph equipped with a reverse-phase column (100 Superspher 100-RP-18; 250 × 4 mm i.d., 5 µm particle size) and a refractive index detector. The methodologies followed for the analysis and for tentative peak identification have been previously described [35, 36]. The total HPLC run time was 40 min. The samples were assayed for their acylglycerol profile without any pretreatment.

For each sample, up to 25 peaks corresponding to the various groups of acylglycerols were separated according to their equivalent carbon number (ECN) [37]. Table 1 presents a tentative identification of the individual peaks observed in the chromatograms, as a function of their ECN and based on the original composition of the fats and oils used and on the predictable formation of new acylglycerols. Only the initial and interesterified samples, corresponding to the star points of the CCRD (experiments number 18–24), corresponding to

the extreme conditions tested, and the centre point (experiments 25–27) were analysed in terms of their acylglycerol profiles (Table 2).

2.2.5 Statistical analysis

The results of the CCRD experiments were analysed using the software Statistica™, version 5, from Statsoft, USA. The linear and quadratic effects of each of the four factors under study (PS and “EPAX 4510TG” concentrations, reaction time and temperature), as well as their linear interactions, on inter-esterification reaction, hydrolysis and oxidation kinetics were calculated. Their significance was evaluated by analysis of variance. The use of five levels for each factor (Table 2) enables the fitting of second-order polynomials to the experimental data points and, therefore, to fit curved surfaces to the experimental data. Thus, a surface, described by a first- or a second-order polynomial equation, was fitted to each set of experimental data points (SFC_{10 °C}, SFC_{20 °C}, SFC_{30 °C} and SFC_{35 °C}, FFA, Abs_{232nm}, and Abs_{270nm} of the interesterified fat blends). First- and second-order coefficients were generated by regression analysis. The goodness-of-fit of these

Table 1. Tentative identification of the individual peaks observed in the chromatograms, as a function of their ECN and fatty acid composition. Fatty acids: C, capric (10:0); Ca, caprylic (8:0); D, DHA (22:6); E, EPA (20:5); L, linoleic (18:2); La, lauric (12:0); Ln, linolenic (18:3); M, myristic (14:0); O, oleic (18:1); P, palmitic (16:0); S, stearic (18:0); DAG, diacylglycerol; EPAX, non-identified peaks from “EPAX 4510TG”.

Peak no.	ECN	Approximate retention time [min]	Initial samples	Final samples
1	?	3.3	EPAX + DAG	EPAX + DAG
2	?	3.9	EPAX + DAG	EPAX + DAG
3	28	4.5	LaCaCa + DAG	LaCaCa + DAG + EDCa
4	28	5.0	CCCa + DAG	CCCa + DAG
5	30	5.8	CCC + DAG + EEE + DDD + EED + DDE	CCC + DAG + EEE + DDD + EED + DDE + EEC + (C22:5) ECa
6	32	6.3	LaCC + CaLaLa	DAG + LaCC + CaLaLa + LnDC
7	34	7.2	LaLaC + CLaM	DAG + LaLaC + LDC + LnLaE
8	36	8.2	LaLaLa	DAG + LaLaLa + DOC + ELLa
9	38	9.5	LaLaM	LaLaM + EOLa + PLaD + SDC + MMD
10	40	10.7	LaLaO	LaLaO + LaES
11	40	11.2	LaMM	LaMM + PEM
12	42	12.5	LaOM	LaOM
13	42	13.3	LaPM	LaPM
14	44	14.3	LaOO	LaOO
15	44	15.1	LaOP	LaOP
16	44	15.9	LaPP + MOM	LaPP + MOM
17	46	16.8	MOO + OOL + PLO	MOO + OOL + PLO
18	46	17.7	MOP + PLP	MOP + PLP
19	48	19.2	OOO	OOO
20	48	20.5	POO	POO
21	48	21.6	POP	POP
22	48	23.0	PPP	PPP
23	50	24.9	OOS	OOS
24	50	26.3	POS	POS
25	50	28.0	PPS	PPS

Table 2. CCRD followed in the experiments as a function of reaction time (*t*), temperature (*T*), PS and “EPAX 4510TG” concentrations used and respective values of SFC_{10 °C}, SFC_{20 °C}, SFC_{30 °C}, and SFC_{35 °C} of fat blends, before (initial) and after enzymatic interesterification (final).

Experiment no.	<i>t</i> [min]	<i>T</i> [°C]	PS [%]	EPAX 4510TG [%]	SFC _{10 °C} initial [%]	SFC _{10 °C} final [%]	SFC _{20 °C} initial [%]	SFC _{20 °C} final [%]	SFC _{30 °C} initial [%]	SFC _{30 °C} final [%]	SFC _{35 °C} initial [%]	SFC _{35 °C} final [%]
1	52.5	60	55	10	60.14	52.81	35.33	31.00	20.34	17.21	14.62	12.41
2	52.5	60	55	20	52.65	45.81	33.62	27.70	20.34	15.92	15.14	11.41
3	52.5	60	75	10	64.72	60.14	48.20	40.31	30.64	24.84	23.35	19.34
4	52.5	60	75	20	60.46	55.33	46.26	37.95	30.37	23.35	24.38	18.80
5	52.5	70	55	10	59.91	54.42	34.66	32.40	20.09	17.94	14.13	13.18
6	52.5	70	55	20	52.73	48.21	34.56	30.44	21.15	18.15	16.23	13.40
7	52.5	70	75	10	64.87	62.33	47.85	43.61	30.03	26.96	22.74	20.78
8	52.5	70	75	20	60.82	56.05	46.51	40.30	31.18	25.31	25.29	20.51
9	97.5	60	55	10	57.99	48.34	34.69	28.71	19.33	15.04	13.34	10.20
10	97.5	60	55	20	49.99	43.46	32.52	25.63	18.25	14.24	14.16	9.70
11	97.5	60	75	10	65.53	58.65	47.25	38.46	30.67	22.74	23.20	17.41
12	97.5	60	75	20	59.62	48.56	45.55	33.71	29.23	20.36	23.43	15.92
13	97.5	70	55	10	59.74	52.53	34.81	30.54	19.78	16.55	14.01	11.63
14	97.5	70	55	20	52.74	46.88	32.59	28.81	19.71	16.48	14.09	11.90
15	97.5	70	75	10	65.49	61.34	47.42	41.29	28.98	25.56	22.36	20.15
16	97.5	70	75	20	61.34	55.32	46.85	39.49	31.54	24.81	25.05	19.87
17	30	65	65	15	58.02	53.67	40.27	35.20	24.46	20.80	17.80	15.72
18	120	65	65	15	56.83	52.24	39.31	33.57	23.88	20.03	17.28	14.89
19	75	55	65	15	59.07	52.18	40.05	32.51	24.72	19.02	18.42	14.32
20	75	75	65	15	58.06	54.61	40.56	36.23	24.84	22.11	18.36	16.15
21	75	65	45	15	54.56	46.63	29.84	25.35	15.63	12.97	11.79	8.54
22	75	65	85	15	67.77	61.54	55.54	45.39	37.87	29.42	31.15	23.85
23	75	65	65	5	65.55	60.13	43.53	38.48	27.26	22.28	20.33	16.71
24	75	65	65	25	54.50	49.73	42.42	33.47	26.53	21.41	21.25	16.72
25	75	65	65	15	59.48	54.17	41.48	34.74	25.87	20.92	19.37	15.75
26	75	65	65	15	59.22	54.11	40.86	35.23	26.03	21.43	19.52	16.20
27	75	65	65	15	58.96	54.05	40.25	35.71	26.20	21.93	19.67	16.66

models was evaluated by the determination coefficients (R^2) and adjusted R^2 (R^2_{adj}). High values of both R^2 and R^2_{adj} suggest a good fit of the model to the experimental data points [32, 33]. By partial differentiation of these polynomial models, the optimum point is found. These solutions are called stationary points. Usually, for most practical applications, the identification of the regions of independent variables corresponding to optimal responses may be directly obtained by visual examination of the response surfaces.

3 Results and discussion

3.1 The effect of initial water activity on interesterification kinetics

The immobilised *C. parapsilosis* lipase/acyltransferase was used at different initial water activity values, as catalyst for the interesterification of PS with PK and “EPAX 4510TG” (65 : 20 : 15, wt-%) at 60 °C for 24 h. Interesterification was indirectly followed by the reduction of SFC values at 35 °C

(Fig. 1). The final content of FFA in the reaction medium during the reaction is also presented in Fig. 1.

The highest SFC_{35 °C} reduction was observed when the biocatalyst was used at an a_w of 0.97: Upon 2 h of reaction time, 54% SFC_{35 °C} reduction was observed, and after 24 h, about 70% reduction was achieved. For lower a_w values (0.43–0.8), quasi-equilibrium was attained after 2 h of reaction time with an SFC_{35 °C} reduction between 7 and 13%. The interesterification activity of the immobilised *C. parapsilosis* lipase/acyltransferase in solvent-free medium showed to be repressed at a_w values equal to or lower than 0.8. When the biocatalyst was used at an a_w of 0.97, the SFC_{35 °C} reduction, observed during the interesterification of fat blends, was comparable to the values obtained when commercial immobilised lipases at low a_w values were used in similar systems [18, 38, 39].

The effect of the water activity on the interesterification activity of *C. parapsilosis* lipase/acyltransferase in organic medium is in accordance with the ability of this enzyme to catalyse alcoholysis over hydrolysis in biphasic aqueous/organic media [25, 39]. Subsequent interesterification experiments were carried out using the immobilised catalyst at an initial a_w of 0.97.

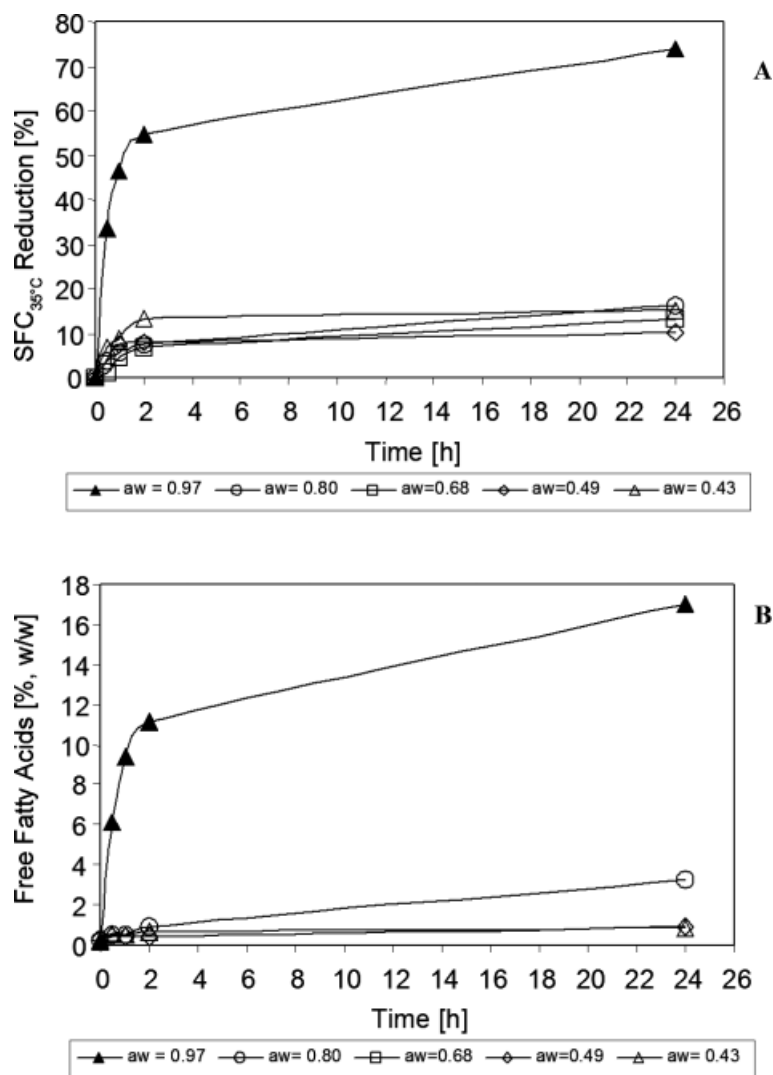


Figure 1. Time course of interesterification catalysed by immobilised *C. parapsilosis* lipase/acyltransferase at different initial a_w values: SFC_{35°C} reduction (A) and FFA content of reaction medium (B).

High esterification and interesterification activities of lipases in non-aqueous media at high water activity values are also reported. That is the case for the lipase from *C. rugosa*, which is known to be sensitive to low-water activity conditions [20]. When this lipase was immobilised in hydrophilic polyurethane foams and used as catalyst for the glycerolysis of olive residue oil in organic media (*n*-hexane), the highest yield of partial acylglycerols [monoacylglycerols (MAG) and diacylglycerols (DAG)] was observed at an initial water activity of 0.83 [22]. Also, when this biocatalyst was used for the esterification of ethanol with butyric acid in *n*-hexane, the highest ester yields were obtained at an a_w of 0.95 [23].

The decrease in SFC_{35°C} values was accompanied by the release of FFA into the reaction medium during interesterification (Fig. 1). FFA may come from the first step of the interesterification reaction or may be produced by hydrolysis of fats in the presence of water molecules [11, 12, 17].

Similar levels of FFA (8%) were observed in batch interesterification of palm olein in water-saturated hexane catalysed by immobilised *C. rugosa* lipase [40]. Values from 5 to 7% and 1.9 to 3.9% were reported respectively when the immobilised *Rhizomucor miehei* lipase ("Lipozyme IM") was used as a catalyst for the interesterification of PS with PK [41] and when the immobilised *C. antarctica* lipase ("Novozyme 435"), at an initial a_w of 0.1, was used in the interesterification of PS with soybean oil and "EPAX 2050TG" [18]. Zainal and Yusoff [42] reported FFA contents between 2.0 and 2.9% in interesterified blends of PS with PK catalysed by "Lipozyme IM" when dried molecular sieves were added to the reaction medium to reduce the water content. The formation of FFA is not desirable since a low yield of interesterified products is obtained and the formation of off-flavours (rancidity) by oxidation of FFA may occur. The addition of molecular sieves also reduced the extent of hydrolysis during the inter-

esterification reaction of corn oil with tristearin, in solvent-free medium, catalysed by a commercial immobilised preparation of *Thermomyces lanuginosa* lipase (“Lipozyme TL IM”) or by this lipase immobilised on octyl-silica [9]. FFA contents lower than 1% were achieved in batch-interesterified blends of anhydrous milk fat, linseed and rapeseed oils, catalysed by “Lipozyme TL IM”, when the water was removed from the biocatalyst during three preliminary consecutive batches where hydrolysis occurred [43]. Also, a decrease in FFA content to values lower than 1 wt-% in the interesterified blends was reported during lipase-catalysed continuous interesterification of fat blends [10, 37, 38, 44], and during batch reutilisation of the biocatalysts [45–47].

3.2 Modelling interesterification

3.2.1 Modification of SFC by lipase/acyltransferase-catalysed interesterification

The reaction conditions, the composition of each fat blend (reaction medium) and the corresponding SFC values at 10,

20, 30 and 35 °C after and before interesterification are presented in Table 2. The FFA content and absorbance values at 232 and 270 nm (related to the presence of initial and final oxidation products, respectively) of the initial blends and interesterified fat blends obtained in the same experiments are shown in Table 3.

In all the experiments, the interesterification catalysed by the immobilised *C. parapsilosis* enzyme promoted a decrease in SFC values of the fat blends at 10, 20, 30 and 35 °C. Reductions in SFC values were as follows: from 4 to 19%, at 10 °C; from 7 to 26%, at 20 °C; from 10 to 30%, at 30 °C; and from 7 to 32%, at 35 °C. Except for the SFC values at 10 °C, the range for the percentage of SFC reduction was similar.

A decrease in the SFC values of the blends upon lipase-catalysed interesterification, in solvent-free media, was also reported [17, 18, 38, 39, 42, 47–50] even when the reaction was conducted under high pressure [35].

In order to investigate the role of the original variables (time, t , temperature, T , and fat blend formulation) on the interesterification kinetics catalysed by *C. parapsilosis* lipase/

Table 3. CCRD as a function of reaction time (t), temperature (T), PS and “EPAX 4510TG” concentrations used and respective values of FFA and absorbance at 232 nm (Abs_{232nm}) and 270 nm (Abs_{270nm}), related to initial and final oxidation products of fat blends, before (initial) and after enzymatic interesterification (final).

Experiment no.	t [min]	T [°C]	PS [%]	EPAX 4510TG [%]	FFA initial [%]	FFA final [%]	Abs_{232nm} initial [%]	Abs_{232nm} final [%]	Abs_{270nm} initial [%]	Abs_{270nm} final [%]
1	52.5	60	55	10	0.23	3.22	5.33	5.71	0.95	1.01
2	52.5	60	55	20	0.33	3.40	9.52	7.13	1.19	1.18
3	52.5	60	75	10	0.54	4.19	3.06	5.07	0.73	0.94
4	52.5	60	75	20	0.32	3.32	10.20	8.49	1.25	1.10
5	52.5	70	55	10	1.01	1.81	5.49	4.85	0.95	0.91
6	52.5	70	55	20	0.32	1.84	9.59	8.51	1.19	1.08
7	52.5	70	75	10	0.33	2.04	9.59	5.55	1.14	1.12
8	52.5	70	75	20	0.33	2.05	7.07	7.43	1.14	1.10
9	97.5	60	55	10	0.47	4.86	6.86	5.09	1.21	1.11
10	97.5	60	55	20	0.22	5.25	8.93	7.75	1.08	1.19
11	97.5	60	75	10	0.33	6.60	5.89	5.01	1.37	1.08
12	97.5	60	75	20	0.34	5.90	9.03	8.25	1.24	2.37
13	97.5	70	55	10	0.30	2.96	6.30	6.23	0.94	1.11
14	97.5	70	55	20	0.35	2.15	9.07	8.63	1.16	1.09
15	97.5	70	75	10	0.32	2.30	5.47	5.92	1.14	1.15
16	97.5	70	75	20	0.42	2.36	9.52	9.21	1.26	1.16
17	30	65	65	15	0.44	2.60	7.71	6.83	1.14	1.12
18	120	65	65	15	0.32	2.67	7.40	6.71	2.28	1.12
19	75	55	65	15	0.33	5.17	7.76	6.47	1.12	1.06
20	75	75	65	15	0.42	1.99	7.41	6.59	1.10	0.95
21	75	65	45	15	0.33	3.15	6.61	6.65	1.16	0.84
22	75	65	85	15	0.32	3.82	8.42	6.82	1.29	1.24
23	75	65	65	5	0.32	5.16	8.48	3.56	1.02	0.83
24	75	65	65	25	0.43	1.28	10.73	10.02	1.17	1.18
25	75	65	65	15	0.32	3.33	6.81	6.51	1.05	1.08
26	75	65	65	15	0.42	3.26	3.14	3.17	0.97	0.96
27	75	65	65	15	0.34	3.19	7.73	6.80	1.19	1.20

acyltransferase, linear and quadratic effects of each variable (factor) and their linear interactions on the final SFC_{10 °C}, SFC_{20 °C}, SFC_{30 °C} and SFC_{35 °C} values were calculated (Table 4).

Since the extent of interesterification is related to the decrease in SFC values, a positive (negative) linear effect of a variable on the SFC value indicates that the increase of this variable leads to a decrease (increase) in the interesterification activity of the biocatalyst. A quadratic positive or negative effect indicates respectively that a concave or a convex surface can be fitted to the response to that variable.

All the variables showed significant positive (temperature and PS concentration) or negative (time and “EPAX 4510TG” concentration) linear effects on all the SFC values of the interesterified fat blends. The interactions ($t \times T$) and ($T \times \text{PS}$) showed important positive effects on the majority of the SFC values. Also, the interactions ($T \times \text{“EPAX 4510TG”}$) and ($\text{PS} \times \text{“EPAX 4510TG”}$) cannot be neglected for SFC_{20 °C} and SFC_{30 °C}, respectively. Quadratic negative effects of time and temperature on the SFC values at 20, 30 and 35 °C were also observed, indicating a convex response surface.

The incorporation, in the starting blends, of high percentages of PS, rich in saturated TAG, is thus a constraint to the SFC_{35 °C} reduction upon interesterification. High SFC_{35 °C} values of interesterified blends rich in PS were also previously reported [17, 18, 38]. In addition, blend formulations containing high amounts of TAG rich in *n*-3 PUFA and/or longer reaction times led to interesterified samples with the lowest SFC values.

The interesterification activity of *C. parapsilosis* lipase/acyltransferase decreased with reaction temperature, suggesting a possible thermal inactivation of the biocatalyst. Con-

versely, temperature within the range 60–80 °C showed a positive effect on the interesterification of fat blends rich in *n*-3 PUFA, catalysed in solvent-free media by “Novozym 435” [18, 38] and also by “Lipozyme TL IM” [10, 17]. The rate of interesterification reaction of tallow with canola oil, catalysed by the commercial immobilised lipase from *Rhizomucor miehei* (Lipozyme IM), was not much influenced by temperature between 50 and 70 °C [49].

In addition, response surfaces, described by polynomial equations, were fitted to the experimental results to visualise the dependence of the responses on the significant variables and therefore to identify the optimal operation conditions.

The significant effects ($p < 0.05$) and those having a confidence range smaller than the value of the effect, or smaller than the standard deviation (data not shown), were included in the model equations of these surfaces. According to Haaland [33], it is better to accept p values higher than 0.05 rather than to take the chance of missing an important factor. The high values of R^2 and R^2_{adj} of these models (Table 5) show a close agreement between the experimental results and the theoretical values predicted by these models [33].

The SFC values of the interesterified fat blends could be fitted to five-dimensional response surfaces (Figs. 2, 3) described by second-order polynomial models as a function of the reaction time, t , temperature, T , PS and “EPAX 4510TG” concentrations (Table 5). The five-dimensional surfaces obtained are illustrated by sets of two three-dimensional surfaces, where only two of the four initial factors vary while keeping the remaining two factors constant and equal to the value assumed in the central point.

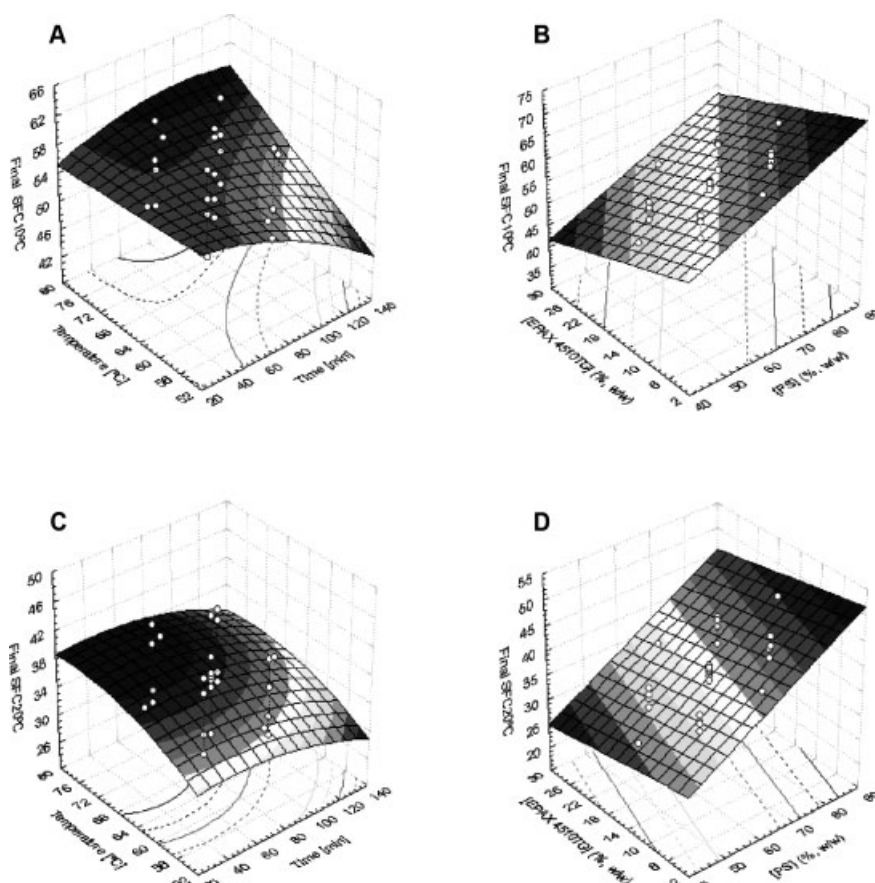
For the SFC response surfaces, no optimal points were observed inside the considered experimental region. Thus, only the identification of the regions corresponding to the best

Table 4. Effects and respective p levels (values between brackets) of reaction time (t), temperature (T), PS and “EPAX 4510TG” concentrations used in CCRD and respective linear interactions on the values of SFC_{10 °C}, SFC_{20 °C}, SFC_{30 °C}, and SFC_{35 °C} and of FFA and absorbance at 232 and 270 nm (Abs_{232nm} and Abs_{270nm}) of fat blends upon enzymatic interesterification.

Factor	Final SFC _{10 °C}	Final SFC _{20 °C}	Final SFC _{30 °C}	Final SFC _{35 °C}	Final FFA	Final Abs _{232nm}	Final Abs _{270nm}
t (linear term)	−1.906 (0.005)	−1.695 (0.0004)	−1.288 (0.002)	−1.226 (0.001)	0.887 (0.023)	0.258 (0.522)	0.153 (0.154)
t (quadratic term)	−0.813 (0.195)	−0.630 (0.115)	−0.728 (0.056)	−0.600 (0.079)	−0.257 (0.490)	0.670 (0.133)	0.063 (0.565)
T (linear term)	2.403 (0.001)	2.572 (8.8E−6)	2.020 (4.4E−5)	1.656 (0.0001)	−2.132 (4.1E−05)	0.341 (0.401)	−0.123 (0.244)
T (quadratic term)	−0.590 (0.338)	−0.638 (0.111)	−0.653 (0.082)	−0.635 (0.065)	0.215 (0.561)	0.549 (0.210)	0.006 (0.955)
PS (linear term)	7.9 (7.2E−9)	9.996 (2.1E−12)	7.944 (1.3E−11)	7.466 (8.6E−12)	0.383 (0.282)	0.112 (0.779)	0.178 (0.102)
PS (quadratic term)	−0.245 (0.685)	−0.139 (0.715)	−0.338 (0.345)	−0.156 (0.627)	0.167 (0.651)	0.651 (0.143)	0.024 (0.824)
EPAX 4510TG (linear term)	−6.0 (1.6E−7)	−2.692 (5.6E−6)	−0.829 (0.025)	−0.297 (0.333)	−0.788 (0.039)	2.909 (7.9E−06)	0.213 (0.056)
EPAX 4510TG (quadratic term)	0.176 (0.772)	0.164 (0.666)	−0.013 (0.971)	0.106 (0.741)	0.037 (0.921)	0.680 (0.127)	0.005 (0.966)
$t \times T$	1.269 (0.088)	0.480 (0.285)	0.497 (0.235)	0.551 (0.152)	−0.804 (0.077)	0.494 (0.323)	−0.153 (0.238)
$t \times \text{[PS]}$	0.007 (0.991)	−0.170 (0.699)	−0.010 (0.980)	0.112 (0.761)	0.076 (0.859)	0.043 (0.930)	0.147 (0.256)
$t \times \text{[EPAX 4510TG]}$	−0.293 (0.675)	−0.053 (0.903)	0.026 (0.948)	−0.050 (0.891)	−0.051 (0.905)	0.152 (0.757)	0.109 (0.393)
$T \times \text{[PS]}$	0.092 (0.895)	0.637 (0.163)	0.581 (0.169)	0.432 (0.254)	−0.411 (0.343)	−0.156 (0.751)	−0.083 (0.512)
$T \times \text{[EPAX 4510TG]}$	0.324 (0.643)	0.584 (0.198)	0.462 (0.267)	0.432 (0.254)	0.036 (0.933)	0.062 (0.900)	−0.197 (0.135)
$\text{[PS]} \times \text{[EPAX 4510TG]}$	−0.433 (0.538)	−0.268 (0.544)	−0.540 (0.199)	−0.196 (0.596)	−0.164 (0.701)	0.212 (0.666)	0.129 (0.314)

Table 5. Model equations for the response surfaces fitted to the values of SFC_{10 °C}, SFC_{20 °C}, SFC_{30 °C}, and SFC_{35 °C}, FFA and absorbance at 232 nm (Abs_{232nm}) of interesterified fat blends as a function of reaction time (*t*), temperature (*T*), PS and “EPAX 4510TG” concentration and respective *R*² and *R*_{adj}².

Model equations	<i>R</i> ²	<i>R</i> _{adj} ²
Final SFC _{10 °C} = 48.239 – 0.308 <i>t</i> – 0.0007 <i>t</i> ² – 0.183 <i>T</i> + 0.396[PS] – 0.598[EPAX 4510TG] + 0.006 <i>tT</i>	0.961	0.949
Final SFC _{20 °C} = –26.740 + 0.057 <i>t</i> – 0.0006 <i>t</i> ² + 1.342 <i>T</i> – 0.013 <i>T</i> ² + 0.086[PS] – 1.029[EPAX 4510TG] + 0.006 <i>T</i> [PS] + 0.012 <i>T</i> [EPAX 4510TG]	0.985	0.979
Final SFC _{30 °C} = –65.697 – 0.077 <i>t</i> – 0.0006 <i>t</i> ² + 1.130 <i>T</i> – 0.011 <i>T</i> ² – 0.101[PS] + 0.268[EPAX 4510TG] + 0.002 <i>tT</i> + 0.006 <i>T</i> [PS] – 0.005[PS][EPAX 4510TG]	0.980	0.968
Final SFC _{35 °C} = –60.261 – 0.099 <i>t</i> – 0.0006 <i>t</i> ² + 1.599 <i>T</i> – 0.012 <i>T</i> ² + 0.373[PS] – 0.030[EPAX 4510TG] + 0.002 <i>tT</i>	0.978	0.970
Final FFA = –1.786 + 0.252 <i>t</i> + 0.055 <i>T</i> + 0.019[PS] – 0.079[EPAX 4510TG] – 0.004 <i>tT</i>	0.793	0.744
Final Abs _{232nm} = 1.981 + 0.0002 <i>t</i> ² + 0.0005 <i>T</i> ² + 5.57E–05[PS] ² + 0.078[EPAX 4510TG] ² + 0.007[EPAX 4510TG] ² – 0.0004 <i>tT</i>	0.817	0.762

**Figure 2.** Response surfaces fitted to the SFC values of interesterified fat blends: SFC at 10 °C as a function of PS and “EPAX 4510 TG” concentrations (A) and of reaction time and temperature (B); SFC at 20 °C as a function of PS and “EPAX 4510 TG” concentrations (C) and of reaction time and temperature (D).

responses was achieved. With respect to medium formulation, the interesterified samples with the lowest SFC values were obtained when low PS and high “EPAX 4510TG” contents were used. Reaction times higher than 90 min and temperatures between 55 and 65 °C led to interesterified blends with lower SFC at 10, 20, 30 and 35 °C.

3.2.2 Production of FFA and oxidation products

Fat blends prior to interesterification have low acidity (0.38% FFA with standard deviation of 0.14). Upon interesterification, an increase in FFA was observed and the final FFA content varied from 1.3 to 6.6% (Table 3).

The linear and quadratic effects of each variable (factor) and their linear interactions on the final FFA content and absorbance values at 232 and 270 nm (Abs_{232nm} and Abs_{270nm}), related to the presence of oxidation products, were calculated (Table 4).

Reaction time and temperature had a linear positive and negative effect, respectively, on the increase in FFA during the interesterification reaction (Table 4). Thus, reactions under higher temperature led to final interesterified fat blends with lower FFA content. Also, the blends obtained at higher reaction temperatures present higher SFC values at 10, 20, 30 and 35 °C, when compared to the samples of similar formulation

interesterified at lower temperature (Table 2). As suggested, a possible inactivation of the biocatalyst may explain the lower interesterification activity at higher temperature.

However, in previous studies, the increase in FFA upon lipase-catalysed interesterification of similar fat blends, in solvent-free media, was shown to be independent of the reaction medium composition, reaction time and temperature [17, 18, 38]. The final FFA values of the interesterified blends can be well fitted to a five-dimensional flat surface (Fig. 4), described by a first-order polynomial model, where the interaction between reaction time and temperature is also important (Table 5). As for SFC values, the FFA response surface is

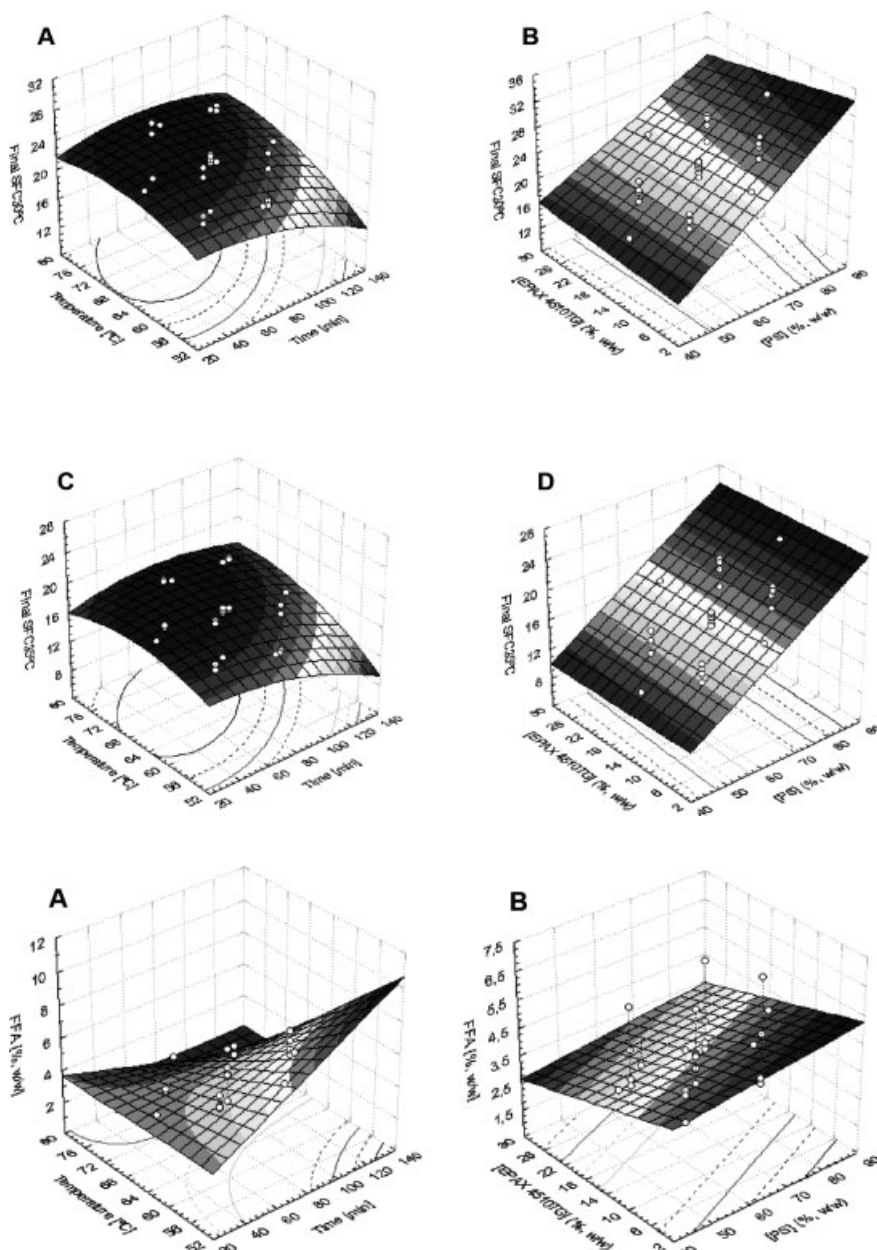


Figure 3. Response surfaces fitted to the SFC values of interesterified fat blends: SFC at 30 °C as a function of PS and "EPAX 4510 TG" concentrations (A) and of reaction time and temperature (B); SFC at 35 °C as a function of PS and "EPAX 4510 TG" concentrations (C) and of reaction time and temperature (D).

Figure 4. Response surfaces fitted to the FFA content of interesterified fat blends as a function of PS and "EPAX 4510 TG" concentrations (A) and of reaction time and temperature (B).

represented by two sets of three-dimensional surfaces, where only two of the four initial factors vary while keeping the remaining two factors constant and equal to the value assumed in the central point (Fig. 4).

Higher FFA contents were observed in samples containing higher amounts of PS and when longer reaction times and lower temperatures were used.

Concerning the absorbance at 232 nm (Abs_{232nm}) related to the presence of primary oxidation products, values from 3.0 to 10.7 were observed in initial fat blends. After interesterification, a decrease in Abs_{232nm} was observed in the majority of fat blends (Table 3). Both reaction time and temperature had considerable quadratic effects on this decrease (Table 4). The “EPAX 4510TG” concentration showed to have linear positive and quadratic effects, while PS concentration had a quadratic effect on the final Abs_{232nm} of the blend. The positive interaction ($t \times T$) cannot be neglected.

The Abs_{232nm} values of the interesterified fat blends can be well described by a second-order polynomial model (Table 5), representing a five-dimensional concave response surface. The lowest Abs_{232nm} values were observed when interesterification was carried out at 55–65 °C for less than 80 min (Fig. 5).

With respect to the absorbance at 270 nm, Abs_{270nm} , related to the presence of final oxidation products responsible for rancid off-flavours, no significant variation was detected during the enzymatic interesterification catalysed by the *C. parapsilosis* enzyme.

When “Lipozyme TL IM” was used as the catalyst for batch interesterification of similar fat blends, no significant effects of the medium composition, reaction time and temperature on the formation of both initial and final oxidation products were detected [17]. However, when “Novozym 435” was used, the reaction time had a linear negative effect on Abs_{232nm} , while the reaction medium composition and/or temperature were significant for the production of final oxidation products [18, 38].

3.2.3 Triacylglycerol profile

As previously reported [36], the modification in the acylglycerol profile is not directly related to the variation in the SFC values at 10, 20, 30 and 35 °C, observed during the enzyme-catalysed interesterification.

As an example, the chromatograms of the fat blend used in experiment number 23 (65% PS, 5% “EPAX 4510TG” and 30% PK), after and before interesterification at 65 °C for 75 min, are presented in Fig. 6. Remarkable modifications on the acylglycerol profile were observed, namely a decrease in the peaks of higher ECN, which is accompanied by an increase in the peaks of medium and low ECN. The observed increase in TAG of medium ECN values confirms the interesterification activity of the *C. parapsilosis* lipase/acyltransferase. The increase in the peaks with low ECN values may correspond to DAG and/or to new TAG species formation (Table 1). The latter are TAG containing long-chain PUFA originally present in the “EPAX 4510TG” concentrate. The presence of DAG in interesterified blends may be beneficial from a technological point of view, in margarine production, due to their emulsifying properties [42].

Experiments 17, 18 and 25 (Table 2) were carried out at the same temperature (65 °C) and using the same fat blend formulation (65% PS, 20% PK, 15% “EPAX 4510TG”), but for different reaction times (30, 120 and 75 min, respectively). The chromatograms of the interesterified blends obtained in these experiments were used to investigate the effect of reaction time on the modification of the acylglycerol profile (Fig. 7). Again, peaks of high ECN value (≥ 48) generally decreased and peaks of medium-to-low ECN value were produced during the reaction catalysed by *C. parapsilosis* lipase/acyltransferase. The main increase was observed for peaks 2 (ECN = 28), 6 (ECN = 34) and 15 (ECN = 44). The production of peaks 6 and 15 occurred mainly during the first 30 min, while peak 2 was produced during the 120-min reaction. The increase in these peaks was accompanied by the decrease of mainly the following peaks: 18 (ECN = 46), 21 and

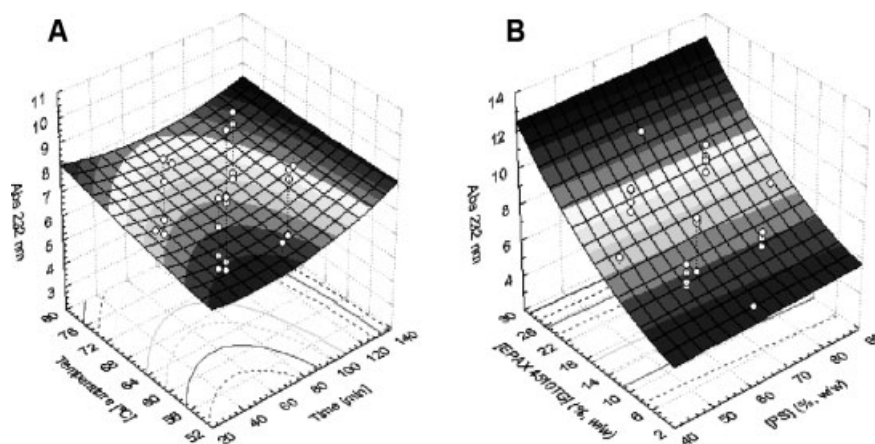


Figure 5. Response surfaces fitted to the absorbance values at 232 nm (related to initial oxidation products) of interesterified fat blends catalysed by *C. parapsilosis* lipase/acyltransferase as a function of PS and “EPAX 4510 TG” concentrations (A) and of reaction time and temperature (B).

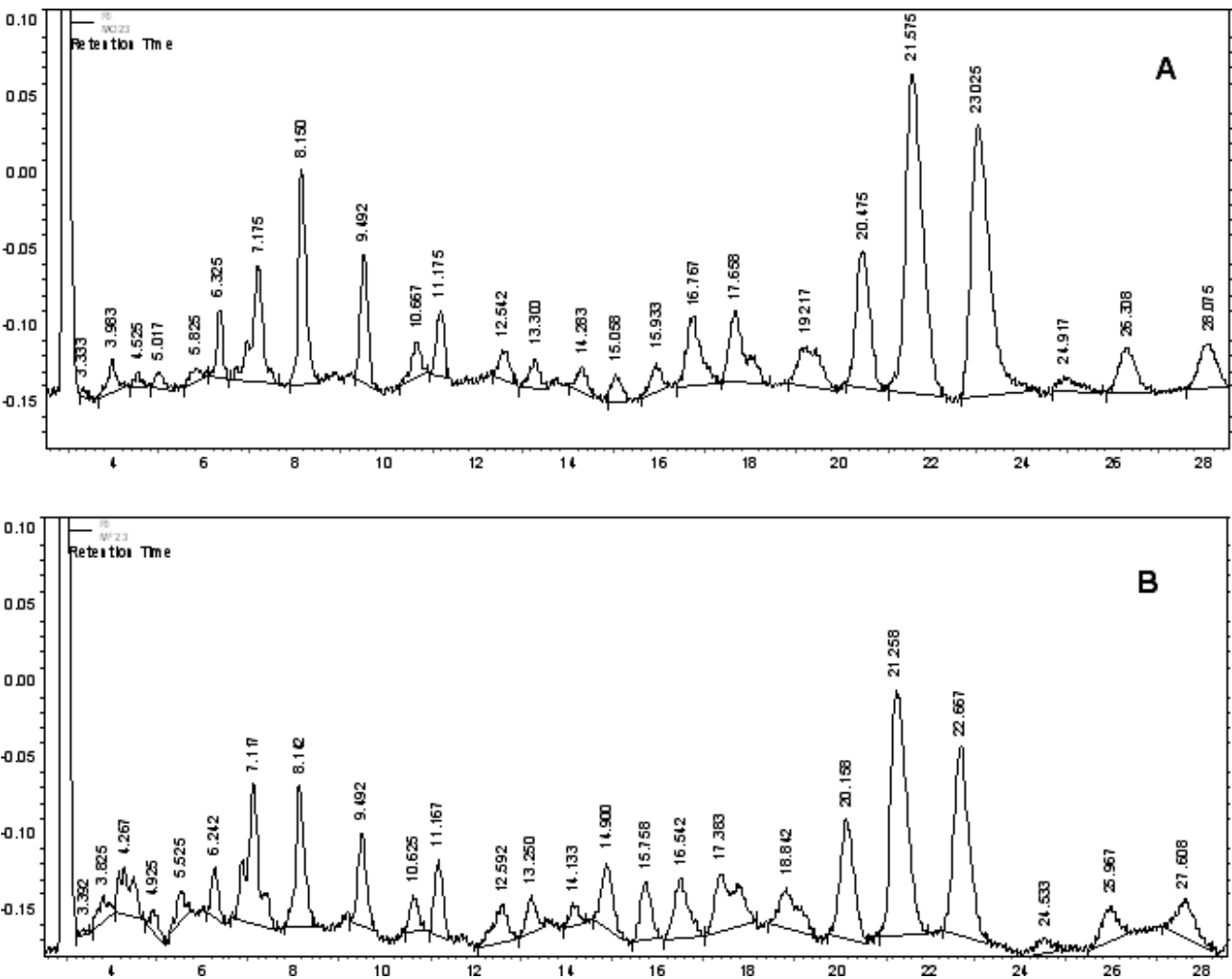


Figure 6. HPLC chromatograms of the acylglycerol profile of the initial fat blend containing 65% PS, 5% “EPAX 4510TG” and 30% PK, after (A) and before (B) interesterification at 65 °C for 75 min, catalysed by *C. parapsilosis* lipase/acyltransferase. A tentative identification and the ECN of the peaks are presented in Table 1.

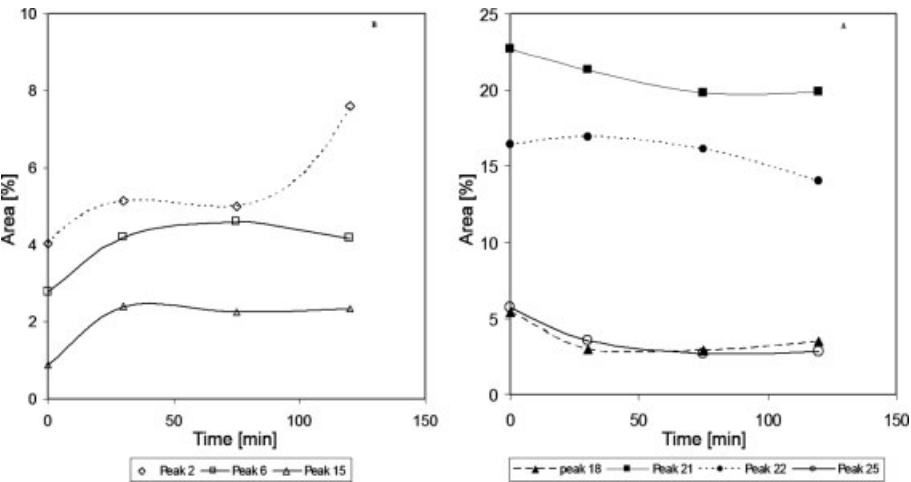


Figure 7. Evolution of the main peaks in the acylglycerol profile of a blend of 65% PS, 20% PK and 15% “EPAX 4510TG”, during the interesterification at 65 °C (samples 17, 18 and 25 of the experimental design – Table 2). A tentative identification and the ECN of the peaks are shown in Table 1.

22 (ECN = 48), and 25 (ECN = 50). Also, the decrease in the amounts of peaks 18 and 25 was observed during the first 30 min of reaction time. Peaks 21 and 22 show an almost linear decrease during the reaction.

In order to investigate the effect of interesterification temperature on the modification of the acylglycerol profile, the chromatograms of the samples obtained in experiments 19, 20 and 25 were compared (Table 2). These experiments were performed with the same fat blend composition (65% PS, 20% PK, 15% “EPAX 4510TG”), for 75 min, and at 55, 75 and 65 °C, respectively.

As in experiments 17, 18 and 25, the main modifications in the acylglycerol profiles of these samples were observed for peaks 2, 6, 7, 15, 16, 18, 21, 22 and 25. The initial blend had the following percentages of these peaks: 3.53% of peak 2; 3.22% of peak 6; 6.03% of peak 7; 1.43% of peak 15; 1.20% of peak 16; 6.88% of peak 18; 22.85% of peak 21; 18.76% of peak 22 and 5.23% of peak 25. Also, the compounds of ECN equal or higher than 46 were consumed (peaks 18, 21, 22 and 25), while peaks 2, 6, 15 and 16 became larger. Peak 7 (ECN = 34) was consumed at 55 °C; at 65 and 75 °C, an increase in the relative amount of this peak was observed.

The reaction temperature seems to affect principally the production of acylglycerols of low and medium ECN (peaks 2, 6, 7, 15 and 16). The variation may be either linear (peaks 6 and 2) or non-linear (peaks 7, 16 and 15). For instance, the highest amount of the compounds corresponding to peak 16 was obtained at 65 °C. Conversely, upon 75 min of interesterification at these temperatures, no variation in the final amounts of each peak of high ECN (peaks 18, 21, 22 and 25) was observed (Fig. 8).

The consumption of TAG species with high ECN, accompanied by the increase of acylglycerol species of lower ECN, has also been observed (i) during the 1,3-selective lipase-catalysed interesterification of fat blends of PS with coconut oil [47], PK [51], canola oil [52], and with PK and TAG enriched in *n*-3 PUFA [35, 36], and (ii) during the chemical interesterification of ternary blends of palm oil, PK

and sunflower oil [53], PS, PK and sunflower oil [54] and PS, PK and TAG enriched in *n*-3 PUFA [36].

4 Conclusions

The immobilised lipase/acyltransferase from *C. parapsilosis* showed, in solvent-free media with a_w as high as 0.97, an interesterification activity similar to that of commercial immobilised lipases at a_w values lower than 0.5.

The best interesterification activity was observed at 55–65 °C, with blends rich in “EPAX 4510TG” concentrate and poor in PS. Also, lower FFA content was observed in blends containing lower PS content. Since these blends have a lower melting point, lower interesterification temperatures can be used. Thus, thermal inactivation of the biocatalyst, observed for temperatures higher than 65 °C, will be avoided. Primary oxidation products in interesterified blends were also minimised for temperatures of 55–65 °C.

This is of much interest for the production of interesterified blends with high content in *n*-3 PUFA for use in nutraceutical applications, particularly as margarine fat bases. The final SFC values at the different temperatures of the interesterified fat blends indicate that these blends can be used to prepare typical base stocks for margarine blends enriched with *n*-3 PUFA. The margarine manufacturer will choose the correct proportions of natural liquid oils and fats and interesterified base stocks, to formulate the requested type of margarine [55]. This will be dictated by (i) the SFC values of the available interesterified fat blends as well as (ii) by the price of the fat components, especially of lauric fats price of which can be higher than that of the other oils ([55], and <http://www.fatsforfoods.com/>, 2008).

Due to the high sensitivity of *n*-3 PUFA to thermal oxidation, these interesterified blends should be preferably used to incorporate in table margarines and spreads or dressings, and should not be used for the production of bakery margarines or frying shortenings.

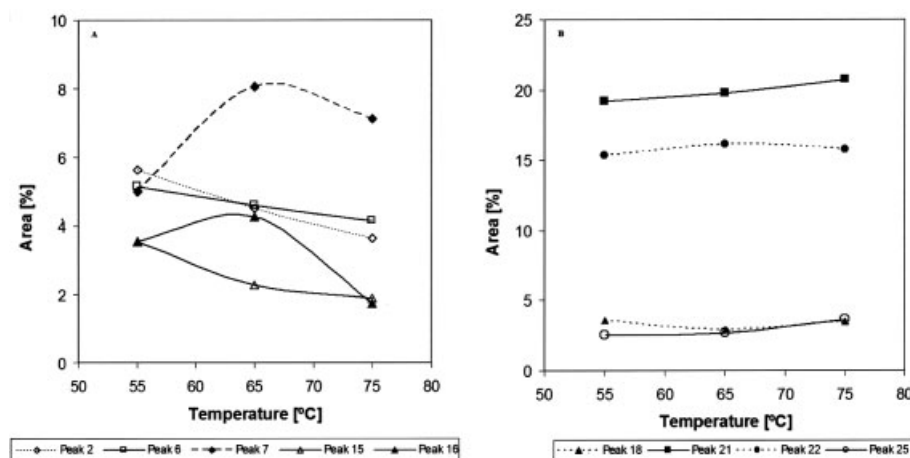


Figure 8. Effect of interesterification temperature on the relative amounts of the main peaks in the chromatograms of a fat blend containing 65% PS, 20% PK and 15% “EPAX 4510TG”, after 75 min of reaction time (samples 19, 20 and 25 of the experimental design – Table 2). A tentative identification and the ECN of the peaks are presented in Table 1.

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Conflict of interest statement

The authors have declared no conflict of interest.

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